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☐ **Brief Communication** ☐

Survival of *Cryptosporidium muris* (strain MCR) oocysts under cryopreservation

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Abstract: We have successfully maintained *Cryptosporidium muris* by cryopreservation. Oocysts were suspended in distilled water, stored at –20°C for 24 hrs, and then cryopreserved at –70°C. Cryopreserved specimens were slowly thawed at 5°C. Oocysts, which had been cryopreserved for 15 months without cryoprotective agents, retained their infectivity by the mouse titration method. Oocysts stored at 5°C in 2.5% potassium dichromate failed to retain their infectivity beyond 6.5 months.

Key words: *Cryptosporidium muris* (strain MCR), cryopreservation, life span

Organisms assigned to the genus *Cryptosporidium* are small coccidian parasite that infect the microvillous region of the mucosal epithelium of vertebrates. Members of the genus *Cryptosporidium* have gained considerable attention since it is recognized as a diarrhea-associated enteropathogen of calves, lambs, and humans (Reese *et al.*, 1982). In immunodeficient persons, particularly those positive for human immunodeficiency syndrome or exogenous immunosuppression, *Cryptosporidium* spp. can cause life-threatening diarrhea.

Conventional methods for storing protozoa consist of preservation at low temperature or continuous biological passage. Biocontainment of protozoa in the laboratory for long-term is difficult. Such maintenance entails infinite labor, time consuming and great expense. Generally, the life span of cryopreserved protozoa is increased considerably. There have been a lot of reports relating to advanced long-term cryopreservation of protozoa, e.g., *Dientamoeba fragilis* (Dwyer and Honigberg, 1971), *Leucocytozoon caulleryi* (Morri *et al.*, 1988) and *Thelohanellus kitauei* (Rhee, 1994).

According to Robertson *et al.* (1992), snap-freezing *Cryptosporidium parvum* oocysts resulted in 100% mortality. Slow freezing, however, was more effective for preserving viable oocysts. After 21 hrs at –22°C, only 33% of the oocysts were viable. Even though the proportion of viable oocysts decreased to < 10% after 152 hrs, a small proportion of oocysts remained viable even after 750 hrs. Prior to the recent work, Rossi *et al.* (1990) described a technique for cryopreservation of *Cryptosporidium* oocysts of calf origin in 5 and 10% dimethyl sulphoxide, without an assessment of viability and infectivity for long-term maintenance.

No satisfactory method has been described to date for long-term *Cryptosporidium* oocyst cryopreservation as far as the authors are aware. Therfore, the technique of cryo-

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preserving oocysts of protozoa is invaluable for maintaining strains in a relatively unaltered state. The described procedure permits prolonged cryopreservation and storage of this microorganism, and possibly other species.

Oocysts of *Cryptosporidium muris* (strain MCR) used in this study were originally isolated from the feces of a naturally infected mouse, *Mus musculus*, in Korea and routinely passed in 3-week-old SPF mice in our laboratory (Rhee *et al.*, 1991a & b). Oocysts were concentrated from the feces by Sheather's sugar floatation method. Preparation and storage of inoculum and enumeration of oocysts for inoculation were done as described previously (Rhee *et al.*, 1995a). Oocysts were less than 1 month old when used for cryopreservation procedure.

Oocysts stored in an aqueous solution of $K_2Cr_2O_7$ were washed with distilled water 3 times and then suspended in distilled water at a concentration of 1×10^7 organisms/ml. Sterile 4 ml screw-cap vials were each dispensed with 1 ml oocyst suspension. For preliminary cryopreservation, the vials involving this sample were transferred to a mechanical freezer at –20°C for 24 hours. Subsequently, the vials were cryopreserved in a deep freezer at –70°C. Prior to transmission experiments to evaluate infectivity of the oocysts, samples were removed from the freezer (–70°C) at 2 month intervals, since 7 months postpreservation and slowly thawed without agitation in a refrigerator at 5°C until the organisms were thoroughly resolved into suspension. After thawing, 10 three-week-old ICR SPF mice were given *per os* a single dose of 2×10^6 thawed oocysts per mouse at each transmission experiment. Fecal examination for oocysts and calculation of the number of oocysts discharged per day (OPD) for each mouse were carried out daily (Rhee *et al.*, 1995a).

Mice infected with 15-month-old cryopreserved oocysts continued to discharge large numbers of oocysts in their feces for a long period, similar to that observed in mice experimentally infected with less than 3-month-old oocysts maintained at 5°C in 2.5% potassium dichromate (Rhee *et al.*, 1995b). That is, oocysts appeared in the feces of mice infected with cryopreserved oocysts 11 days after inoculation, reached a peak at 27 days, and disappeared after 72 days. No significant changes in survival of frozen protozoa were observed in comparison to samples stored < 3 months at 5°C. In addition, frozen oocysts also retained their infectivity after cryopreservation < 15 months.

These results indicate that the novel cryopreservation technique has negligible effect on the infectivity of oocysts, while oocysts suspended in 2.5% aqueous solution of potassium dichromate at 5°C for 6.5 months did not infect 10 age-matched control mice. Also, Sherwood *et al.* (1982) demonstrated that infectivity of a calf *Cryptosporidium* sp. isolate to 1- to 4-day-old mice after storage at 4°C in phosphate-buffered saline or 2.5% potassium dichromate was maintainable for 4 to 6 months. Meanwhile, no infectivity of *C. baileyi* to chicken was detectable after 3 months of storage in 2.5% potassium dichromate (Rhee *et al.*, 1995a). Some researchers (Reese *et al.*, 1982; Robertson *et al.*, 1992; Rossi *et al.*, 1990) have demonstrated that a number of cryopreservative techniques apparently destroy the infectivity of *Cryptosporidium parvum* oocysts for mice. This difference of the infectivities may be due to difference between species of *Cryptosporidium* in resistance to damage by freezing.

Thus, oocysts of *Cryptosporidium muris* (strain MCR) remained viable for 15 months by freezing preservation technique described for the first time, while the protozoa kept at 5°C lost their viability as early as 6.5 months after initial preservation.

REFERENCES

Dwyer DM, Honigberg BM (1971) Freezing and maintenance of *Dientamoea fragilis* in liquid nitrogen. *J Parasitol* **57**(1): 190-191.

Morri T, Matsui T, Iijima T, Abe N, Yoshizawa A, Chikatsune M, Watanabe M (1988) Cryopreservation of *Leucocytozoon caulleryi* sporozoites. *Protozoology* **35**(3): 356-359.

Reese NC, Current WL, Ernst JV, Bailey WS (1982) Cryptosporidiosis of man and calf: a case report and results of experimental infections in mice and rats. *Am J Trop Med Hyg* **31**: 226-229.

Rhee JK (1994) Supplemental knowledge on survival of *Thelohanellus kitauei* spores *in vitro*. *Korean J Parasitol* **32**(1): 57-59.

Rhee JK, Seu YS, Park BK (1991a) Isolation and identification of *Cryptosporidium* from various animals in Korea I. Prevalence of *Cryptosporidium* in various animals. *Korean J Parasitol* **29**(2): 139-148.

Rhee JK, Seu YS, Park BK (1991b) Isolation and identification of *Cryptosporidium* from various animals in Korea II. Identification of *Cryptosporidium muris* from mice. *Korean J Parasitol* **29**(2): 147-159.

Rhee JK, Jang BG, Park BK (1995a) Oocyst production and immunogenicity of *Cryptosporidium baileyi* in chickens and mallards. *Korean J Parasitol* **33**(1): 45-54.

Rhee JK, Yook SY, Park BK (1995b) Oocyst production and immunogenicity of *Cryptosporidium muris* (strain MCR) in mice. *Korean J Parasitol* **33**(4): 377-382.

Robertson LJ, Campbell AT, Smith HV (1992) Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. *Appl Environ Microbiol* **58**(11): 3494-3500.

Rossi P, Pozio E, Besse MG (1990) Cryopreservation of *Cryptosporidium* sp. oocysts. *Trans R Soc Trop Med Hyg* **84**: 68.

Sherwood D, Angus KW, Snodgrass DR, Tzipori S (1982) Experimental cryptosporidiosis in laboratory mice. *Infect Immun* **38**(2): 471-475.

=초록=

쥐와포자충(MCR주)의 냉동 보존

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쥐와포자충(MCR주)을 장기간에 걸쳐 그 생명을 유지시키기 위하여 냉동 보존을 시도하였다. 그 오오시스트를 냉동보호제를 첨가하지 않고 증류수에 현탁시켜 −20°C에서 24시간 예비냉각시킨 다음 −70°C에 냉동보존하였다. 냉동 오오시스트를 5°C에서 해동시켜 마우스에 접종시킨 바 15개월간 냉동보존시켜도 사멸하지 않고 마우스에의 감염성이 유지되었다. 한편, 2.5% 중크롬산칼륨에 현탁시켜 6.5개월간 5°C에 냉장한 오오시스트는 이미 사멸하여 감염성이 소실되었다.

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